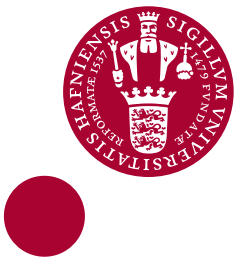

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RESEARCH ARTICLE

Helicobacter pylori VacA Suppresses *Lactobacillus acidophilus*-Induced Interferon Beta Signaling in Macrophages via Alterations in the Endocytic Pathway

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ABSTRACT ***Helicobacter pylori*** **causes chronic gastritis and avoids elimination by the immune system of the infected host. The commensal bacterium** ***Lactobacillus acidophilus*** **has been suggested to exert beneficial effects as a supplement during** ***H. pylori*** **eradication therapy. In the present study, we applied whole-genome microarray analysis to compare the immune responses induced in murine bone marrow-derived macrophages (BMDMs) stimulated with** ***L. acidophilus*****,** ***H. pylori*****, or both bacteria in combination. While** ***L. acidophilus*** **induced a Th1-polarizing response characterized by high expression of interferon beta (IFN-β) and interleukin 12 (IL-12),** ***H. pylori*** **strongly induced the innate cytokines IL-1β and IL-1α. In BMDMs prestimulated with** ***L. acidophilus*****,** ***H. pylori*** **blocked the expression of** ***L. acidophilus*****-induced IFN-β and IL-12 and suppressed the expression of key regulators of the Rho, Rac, and Cdc42 GTPases. The inhibition of** ***L. acidophilus*****-induced IFN-β was independent of** ***H. pylori*** **viability and the virulence factor CagPAI; however, a vacuolating cytotoxin (*vacA*) mutant was unable to block IFN-β. Confocal microscopy demonstrated that the addition of** ***H. pylori*** **to** ***L. acidophilus*****-stimulated BMDMs redirects intracellular processing, leading to an accumulation of** ***L. acidophilus*** **in the endosomal and lysosomal compartments. Thus, our findings indicate that** ***H. pylori*** **inhibits the development of a strong Th1-polarizing response in BMDMs stimulated with** ***L. acidophilus*** **by blocking the production of IFN-β in a VacA-dependent manner. We suggest that this abrogation is caused by a redirection of the endocytotic pathway in the processing of** ***L. acidophilus*****.**

IMPORTANCE **Approximately half of the world's population is infected with** ***Helicobacter pylori*****. The factors that allow this pathogen to persist in the stomach and cause chronic infections have not yet been fully elucidated. In particular, how** ***H. pylori*** **avoids killing by macrophages, one of the main types of immune cell underlying the epithelium, remains elusive. Here we have shown that the** ***H. pylori*** **virulence factor VacA plays a key role by blocking the activation of innate cytokines induced by the probiotic** ***Lactobacillus acidophilus*** **in macrophages and suppresses the expression of key regulators required for the organization and dynamics of the intracellular cytoskeleton. Our results identify potential targets for the treatment of** ***H. pylori*** **infection and vaccination, since specific inhibition of the toxin VacA possibly allows the activation of an efficient immune response and thereby eradication of** ***H. pylori*** **in the host.**

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The Gram-negative bacterium *Helicobacter pylori* colonizes the human stomach and has infected more than half of the world's population (1, 2). Upon infection, *H. pylori* activates an inflammatory response which leads to the recruitment of macrophages, neutrophils, and lymphocytes to the gastric tissue (3). Macrophages can efficiently engulf *H. pylori*; however, this pathogenic bacterium has developed mechanisms to avoid killing upon phagocytosis and prevents the induction of an adaptive immune response (4–7). It is likely that the failure of macrophages to eliminate *H. pylori* contributes to bacterial persistence in the host and thereby to the development of chronic infection. Indeed, we have previously reported the requirement for macrophages in the development of *H. pylori*-induced gastritis *in vivo* (8).

It is currently not fully understood how *H. pylori* avoids elimination by the immune system and is able to survive and persist in human macrophages. Several different mechanisms have been suggested based on the virulence factors expressed by *H. pylori* (9). *H. pylori* retards its own uptake by phagocytes, which has been suggested to be dependent on type IV secretion components encoded by the cytotoxin-associated gene pathogenicity island (CagPAI) (10, 11), one of the main pathogenic factors in *H. pylori* infection (12). Once it is intracellular, *H. pylori* actively delays

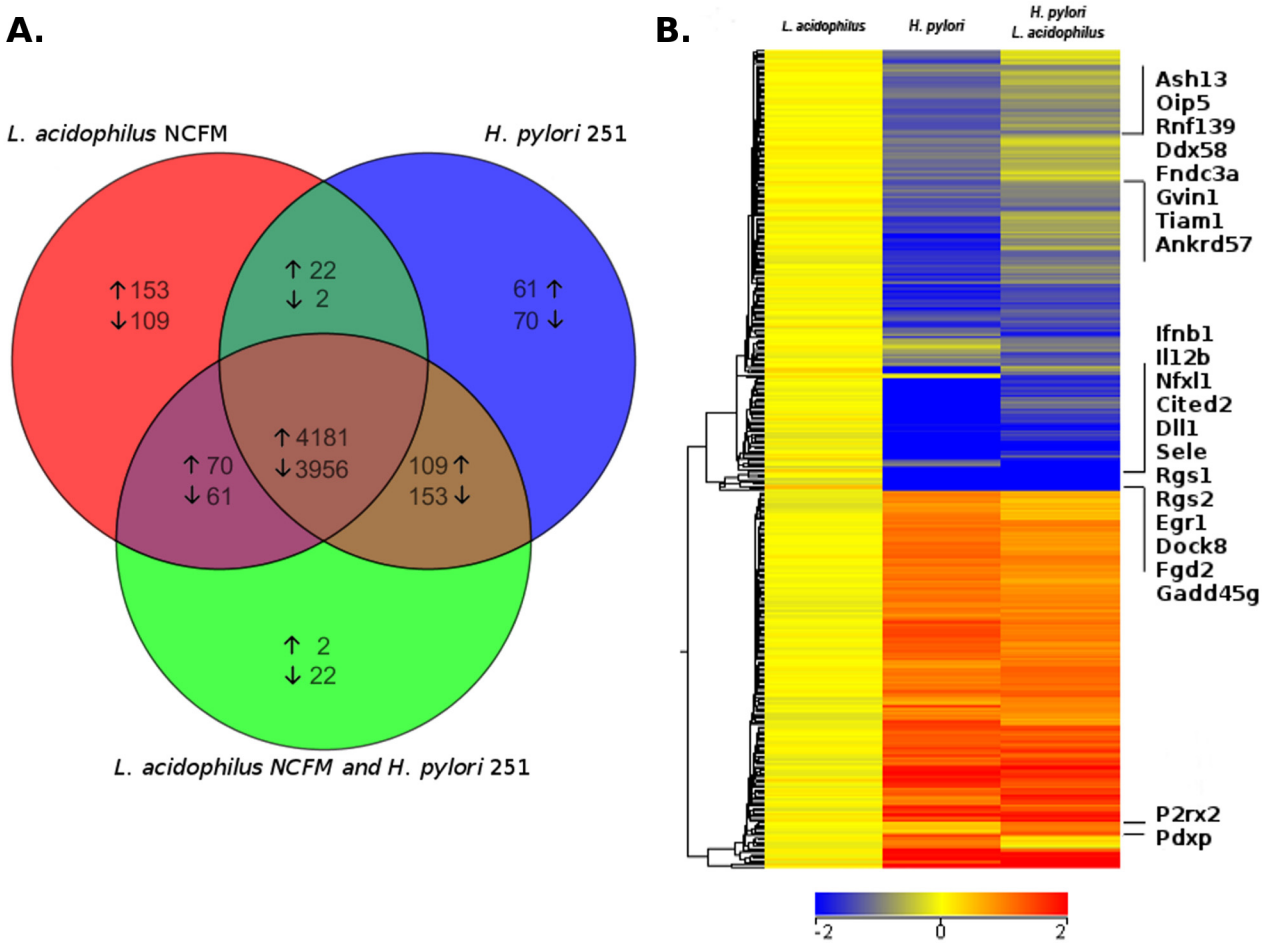

FIG 1 *H. pylori* modulates the stimulatory profile induced by *L. acidophilus* in murine bone marrow-derived macrophages. Murine BMDMs from three individual mice were stimulated for 5 h with either *L. acidophilus* NCFM or *H. pylori* 251 or prestimulated with *L. acidophilus* NCFM for 1 h prior to the addition of *Helicobacter pylori* 251. (A) Venn diagram illustrating pairwise overlap of differentially expressed genes ($P < 0.01$; fold change greater than ± 2) by one-way ANOVA with Benjamini-Hochberg multiple testing correction. (B) Heat map of differentially expressed genes ($P < 0.01$, fold change greater than ± 1.5) expressed at a lower or higher rate than with *L. acidophilus* treatment. Blue designates genes expressed at a lower level than with *L. acidophilus* alone, while red indicates greater expression. Data were classified using hierarchical clustering by genes with Euclidean distances and centroid linkage rules.

actin polymerization and phagosome formation in addition to disrupting membrane trafficking in macrophages (5).

H. pylori has been indicated to utilize several virulence factors facilitating the escape of intracellular degradation in phagocytes. Upon uptake in macrophages, *H. pylori* initially localizes in phagosomes that coalesce into "megasomes" containing multiple bacteria (5, 13), which has been proposed to cause resistance to intracellular killing (5). A study by Zheng and coworkers suggested that retention of TACO, a tryptophan aspartate-containing coat protein on phagosomes, impairs fusion of phagosomes and lysosomes in macrophages infected with *H. pylori* (7).

It has been reported that probiotic supplementation to *H. pylori* eradication therapy increases tolerability and decreases side effects (14–16), but the results are contradictory (17). We have previously shown that the probiotic bacterium *Lactobacillus acidophilus* NCFM is a strong inducer of the Th1-polarizing cytokines interferon beta (IFN-β) and interleukin 12 (IL-12) in dendritic cells (18–20). In this study, we investigated whether *L. acidophilus* is able to modulate the inflammatory response induced by *H. pylori* in bone marrow-derived macrophages (BMDMs). We utilized whole-genome microarray analysis to identify the cellular and molecular mechanisms involved in uptake and processing of the bacteria in addition to the immune response generated. BMDMs were stimulated with either *L. acidophilus* or *H. pylori* or with both bacteria in combination. Contrary to our expectations, our results demonstrate that *H. pylori* is able to block the induction of IFN-β and IL-12 by *L. acidophilus*. Furthermore, *Rgs1/2*, *Fgd2*, and *Dock8*, key regulators of the Rho, Rac, and Cdc42 GTPases, respectively, required for the organization and dynamics of the actin cytoskeleton, were strongly suppressed when *H. pylori* was added to BMDMs prestimulated with *L. acidophilus*. This inhibition was independent of the viability of *H. pylori* or the virulence factor CagPAI but completely dependent on the VacA toxin. Confocal microscopy revealed that the addition of *H. pylori* to *L. acidophilus*-treated BMDMs induces alterations in the endocytic pathway that prevent intracellular processing of *L. acidophilus* and thereby the induction of IFN-β. Thus, *H. pylori* is able to prevent the activation of innate cytokines that can mod-

TABLE 1 Strongest induced/suppressed genes in BMDMs stimulated with *L. acidophilus* and *H. pylori* in combination

Probe name	Annotation	Fold change		Description
		Induced	Suppressed	
A_51_P498526	*P2rx2*	7.68		Purinergic receptor P2X, ligand-gated ion channel 2
A_52_P609120	*Pdxp*	3.90		Pyridoxal (pyridoxine, vitamin B6) phosphatase
A_51_P144180	*Ifnb*		18.47	Interferon beta 1
A_51_P419768	*Rgs2*		11.13	Regulator of G-protein signalling 2
A_51_P478556	*Cited2*		10.42	Cbp/p300-interacting transactivator 2
A_51_P306017	*Dll1*		8.73	Delta-like 1
A_51_P455326	*Sele*		7.97	Selectin, endothelial cell
A_51_P315904	*Gadd45g*		4.46	Growth arrest and DNA-damage-inducible 45 gamma
A_51_P260683	*Rgsl*		4.43	Regulator of G-protein signaling 1
A_51_P385812	*1112b*		4.29	lnterleukin 12b
A_51_P367866	*Egrl*		4.23	Early growth response 1
A_51_P149349	*Nfxl1*		3.72	Nuclear transcription factor, X-box binding-like 1
A_51_P484718	*Dock8*		3.71	Dedicator of cytokinesis 8
A_52_P260994	*Fgd2*		3.66	FYVE, RhoGEF and PH domain containing 2

ulate the adaptive immune response in macrophages via the virulence factor VacA, which possibly allows persistence and limited eradication in the host.

RESULTS

***H. pylori* modulates the stimulatory profile induced by *L. acidophilus* in murine bone marrow-derived macrophages.** To compare the gene expression profiles after stimulation with *L. acidophilus* and *H. pylori*, murine bone marrow-derived macrophages (BMDMs) were stimulated either with *L. acidophilus* NCFM or with an *H. pylori* clinical isolate, strain 251, or with both bacteria in combination. Since we wanted to ensure that *L. acidophilus* is able to interact with the macrophages prior to their encounter with the motile pathogen *H. pylori*, we added *H. pylori* 251 to BMDMs after 1 h of prestimulation with *L. acidophilus* NCFM (cell-bacterium ratio, 1:1). After 5 h, the BMDMs were harvested and genome-wide microarray analysis was performed. In total, 4,181 upregulated and 3,956 downregulated genes were common in the three groups of stimulatory conditions (Fig. 1A). In the *L. acidophilus*-stimulated group, 153 genes were exclusively upregulated and 109 genes exclusively downregulated. Upon stimulation with *H. pylori* alone, 61 genes were exclusively upregulated and 70 genes exclusively downregulated. With both strains in combination, only 2 genes were exclusively upregulated and 22 genes exclusively downregulated. The two groups of BMDMs stimulated with either *L. acidophilus* or *H. pylori* alone had 22 upregulated and 2 downregulated genes in common. In contrast, for *L. acidophilus* stimulation, there were 70 upregulated and 61 downregulated genes, and for *H. pylori*, there were 109 upregulated and 153 downregulated genes, in common with the BMDMs that were stimulated with both bacteria.

The top 20 most strongly upregulated genes in BMDMs stimulated with *L. acidophilus* or *H. pylori* are listed in Table S1 and Table S2 in the supplemental material, respectively. Analysis of the genes expressed revealed that *L. acidophilus* strongly induced innate response genes that promote the development of an adaptive immune response, characterized by IL-12 and IFN-β and the chemokines Cxcl10 and Cxcl11, known to be involved in recruitment of activated T cells to the site of infection (Table S1). In contrast, *H. pylori* induced primarily an innate immune response upregulating the cytokines IL-1β (*Il1b*) and IL-1α (*Il1a*) and the neutrophil-recruiting chemokines (*Cxcl3, Cxcl1,* and *Cxcl2*) (see Table S2). Notably, *H. pylori* strongly induced serum amyloid A 3 (*Saa3*), an acute-phase reactant elevated in chronic low-grade inflammation (21), and *Socs3* (suppressor of cytokine signaling 3), a negative regulator of cytokine signaling (22). Figure S1 illustrates a heat map of the genes differentially expressed upon addition of *H. pylori*, *L. acidophilus*, or both strains in combination compared to expression in nonstimulated cells ($P < 0.01$; fold change of greater than ±2). Figure 1B displays the downregulated genes (blue color) and upregulated genes (red color) induced by *L. acidophilus*, *H. pylori*, or both strains in combination compared to *L. acidophilus* alone ($P < 0.01$; fold change of greater than ±2). Blue designates genes expressed at a lower level than with *L. acidophilus* alone, while red indicates greater expression.

Table 1 and Fig. 1B present genes that were either strongly induced or suppressed upon the addition of both *L. acidophilus* and *H. pylori* to BMDMs relative to results with *L. acidophilus* alone. Only two genes were strongly induced: *P2rx2* (purinergic receptor P2X, ligand-gated ion channel 2) and *Pdxp* (pyridoxal [pyridoxine, vitamin B6] phosphatase), both regulators of actin cytoskeleton dynamics, were upregulated 7.7-fold and 3.9-fold, respectively. P2rx2 belongs to the family of ligand-gated ion channels that open in response to ATP (23). Activation of P2X receptors induces actin cytoskeleton alterations via Rho activation in macrophages (24, 25). Pdxp is located in the cytosol, colocalizes with the actin cytoskeleton, and acts as a positive regulator of actin filament depolymerization (26). In total, 12 genes were strongly suppressed (Table 1). The gene encoding IFN-β (*Ifnb*) was the most significantly inhibited (18.5-fold) over 41,000 genes and transcripts analyzed. Interleukin 12b (encoded by *Il12b*) was likewise significantly suppressed (4.3-fold). Other strongly suppressed genes identified were *Rgs2* and *Rgs1* (regulator of G-protein signalling 2 and 1) (27), *Dock8* (dedicator of cytokinesis 8) (28), and *Fgd2* (FYVE, RhoGEF, and PH domain-containing 2) (29), all involved in signaling of Rho, Rac, or Cdc42 GTPase, which are key regulators of the actin cytoskeleton ($P < 0.01$) (30).

***H. pylori* blocks production of *L. acidophilus*-induced IFN-β and IL-12 in murine bone marrow-derived macrophages.** Our microanalysis data revealed that genes encoding the cytokines IFN-β and IL-12 were strongly inhibited upon the addition of *H. pylori* to BMDMs prestimulated with *L. acidophilus* (Table 1),

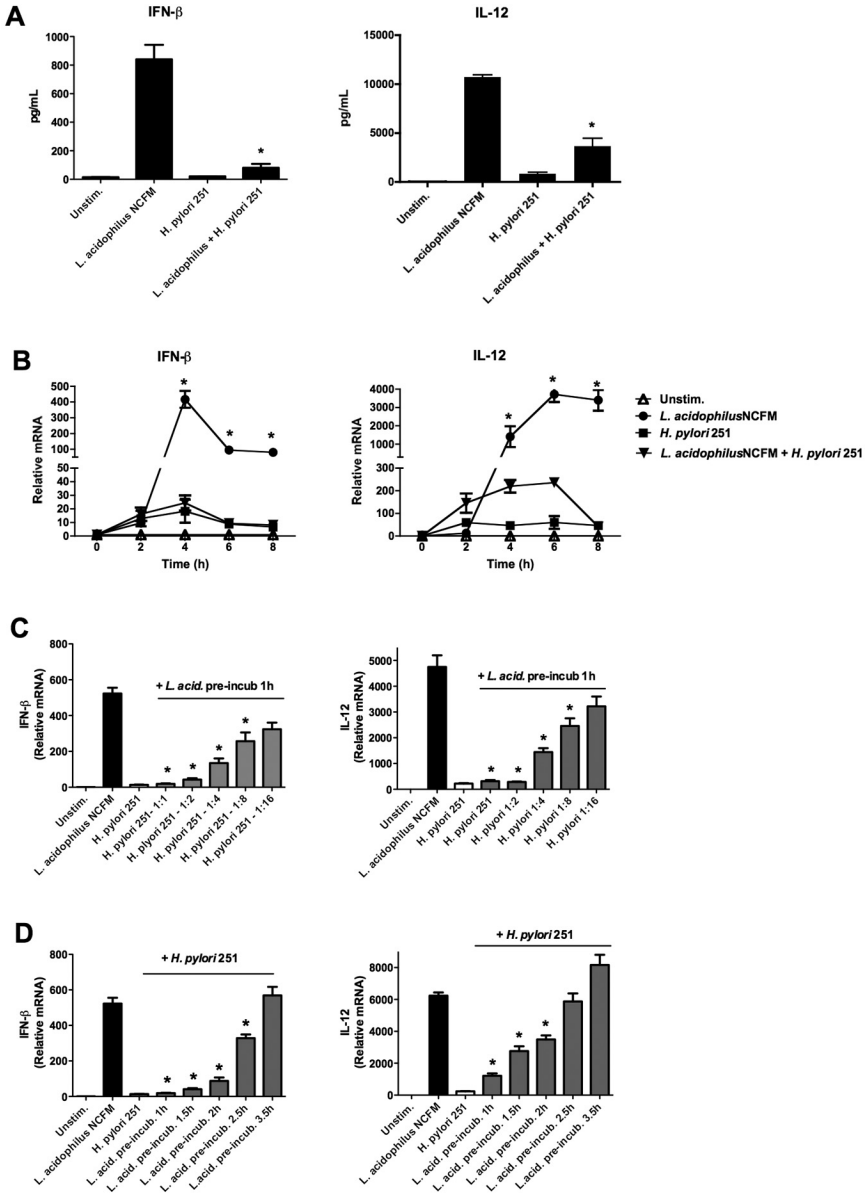

FIG 2 *H. pylori* 251 blocks the induction of IFN-β and IL-12 in murine bone marrow-derived macrophages stimulated with *L. acidophilus*. BMDMs were stimulated with either *L. acidophilus* NCFM or *H. pylori* 251 or prestimulated with *L. acidophilus* NCFM for 1 h prior to addition of *Helicobacter pylori* 251. (A) IFN-β and IL-12 were measured by ELISA in supernatants after 10 h of stimulation (*L. acidophilus* NCFM plus *H. pylori* 251 versus *L. acidophilus* NCFM alone; *, $P < 0.01$). Unstim., unstimulated. (B) RNA was extracted after 2, 4, 6, or 8 h of stimulation, cDNA was synthesized, and the expression of *Ifn-β* and *Il-12* was analyzed by RT-PCR (*L. acidophilus* NCFM plus *H. pylori* 251 versus *L. acidophilus* NCFM alone; *, $P < 0.01$). (C) *H. pylori* 251 was added at various MOI (1:1, 1:2, 1:4, 1:8, and 1:16, *H. pylori*/BMDM ratio) to BMDMs prestimulated with *L. acidophilus* (1 h). RNA was extracted after 4 h, cDNA was synthesized, and the expression of *Ifn-β* and *Il-12* was analyzed by RT-PCR. (D) BMDMs were prestimulated with *L. acidophilus* (L. acid. pre-incub.) for 1 h, 1.5 h, 2 h, 2.5 h, and 3.5 h prior to addition of *H. pylori* 251, RNA was extracted after 4 h, cDNA was synthesized, and the expression of *Ifn-β* and *Il-12* was analyzed by RT-PCR (*L. acidophilus* NCFM plus *H. pylori* 251 versus *L. acidophilus* NCFM alone; *, $P < 0.01$). Data are means and SD for triplicate cultures and are representative of three independent experiments.

hence altering the effect of *L. acidophilus*. To confirm this, BMDMs were stimulated with either *L. acidophilus* NCFM or *H. pylori* 251 or pretreated with *L. acidophilus* NCFM for 1 h prior to the addition of *H. pylori* 251. The levels of the IFN-β and IL-12 proteins were determined in the supernatants after 10 h of stimulation (Fig. 2A). *L. acidophilus* induced high expression of IFN-β (840 pg/ml) and IL-12 (10,700 pg/ml), whereas *H. pylori* 251 induced small amounts of IFN-β and IL-12 (20 pg/ml and 850 pg/ml, respectively). As expected, the addition of *H. pylori* to BMDMs prestimulated with *L. acidophilus* significantly suppressed the release of IFN-β by 90% and that of IL-12 by 66% (*, $P < 0.01$).

To determine whether this inhibition is time dependent, the gene expression of *Ifn-β* and *Il-12* was analyzed by reverse transcription-PCR (RT-PCR) after 2, 4, 6, and 8 h of stimulation (Fig. 2B). After 4 h, the expression of *Ifn-β* in the *L. acidophilus*-stimulated BMDMs reached a maximum of 400-fold, whereas *H. pylori* induced *Ifn-β* only 15-fold compared to results for controls. However, when *H. pylori* was added to BMDMs prestimulated with *L. acidophilus*, the expression of *Ifn-β* was completely blocked, and the expression of *Il-12* was strongly reduced (by 94%) at all time points compared to results for BMDMs stimulated with *L. acidophilus* alone (*, $P < 0.01$), indicating that the inhibitory effect is exerted at a very early stage.

In all of our previous experiments, we added both *L. acidophilus* and *H. pylori* at a multiplicity of infection (MOI) of 1:1 to the BMDMs. We next sought to investigate the potency of *H. pylori* to block the expression of *Ifn-β* and added *H. pylori* to *L. acidophilus*-stimulated cells at MOIs of 1:2, 1:4, 1:8, and 1:16 (*H. pylori*-cell ratio) (Fig. 2C). Even at an MOI as low as 1:8, *H. pylori* was still able to significantly downregulate the *L. acidophilus*-induced *Ifn-β* expression by 51% (*, $P < 0.01$). Only at an MOI of 1:16 the inhibition of *Ifn-β* was no longer significant. A similar effect was observed when the expression of *Il-12* was measured. Since *H. pylori* is able to suppress the induction of *Ifn-β* and *Il-12* at a very low MOI, it is indicated that this inhibitory effect is not restricted to the action of an individual bacterium but rather to a virulence determinant secreted by *H. pylori*.

Next, we aimed to determine the time point at which *H. pylori* is no longer able to suppress the induction of *Ifn-β* and *Il-12* by *L. acidophilus* (Fig. 2D). At a preincubation time of 1.5 h with *L. acidophilus*, *H. pylori* 251 significantly inhibited the expression of *Ifn-β* by 92%, at 2 h by 83%, and at 2.5 h by 63%, compared to results for BMDMs stimulated with *L. acidophilus* alone (*, $P <$

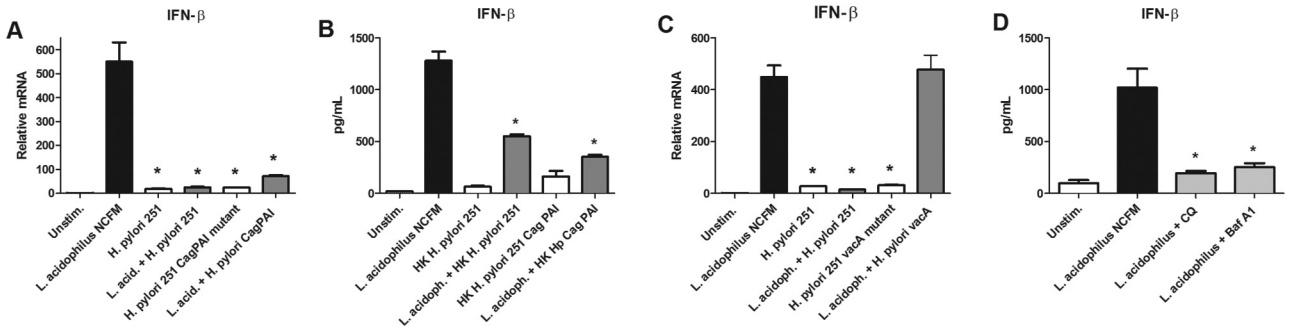

FIG 3 Inhibition of IFN-β by *H. pylori* is independent of viability and the virulence factor CagPAI but dependent on the toxin VacA. (A) BMDMs were stimulated with *L. acidophilus* NCFM, *H. pylori* 251, or the *H. pylori* 251 CagPAI mutant alone or prestimulated with *L. acidophilus* NCFM (L. acid.) for 1 h prior to addition of *H. pylori* strains. RNA was extracted after 4 h, cDNA was synthesized, and the expression of *Ifn-β* was measured by RT-PCR (*L. acidophilus* NCFM plus *H. pylori* 251 or plus the *H. pylori* 251 CagPAI mutant versus *L. acidophilus* NCFM alone; *, $P < 0.01$). (B) BMDMs were prestimulated with *L. acidophilus* NCFM for 1 h prior to addition of heat-killed (HK) *H. pylori* 251 and heat-killed *H. pylori* 251 CagPAI mutant. IFN-β was measured in the supernatants 10 h after stimulation by ELISA (*L. acidophilus* NCFM [L. acidoph.] plus HK *H. pylori* 251 or plus the *H. pylori* 251 CagPAI mutant versus *L. acidophilus* NCFM alone; *, $P < 0.01$). (C) BMDMs were prestimulated with *L. acidophilus* NCFM for 1 h prior to addition of *H. pylori* 251 or the *H. pylori* 251 *vacA* mutant. RNA was extracted after 4 h, cDNA was synthesized, and the expression of *Ifn-β* was measured by RT-PCR (*L. acidophilus* NCFM plus the *H. pylori* 251 *vacA* mutant versus *L. acidophilus* NCFM alone; *, $P < 0.01$). (D) BMDMs were preincubated for 30 min with the endosomal acidification blockers chloroquine (CQ) (final concentration, 20 μM) and bafilomycin A1 (Baf A1) (final concentration, 25 μM) prior to stimulation with *L. acidophilus* NCFM. IFN-β was measured in the supernatants 10 h after stimulation by ELISA (*L. acidophilus* NCFM plus inhibitors versus *L. acidophilus* NCFM alone; *, $P < 0.01$). Represented are the means ± SEM for triplicate macrophage cultures, which are representative of three independent experiments.

0.01). However, a downregulation of *Ifn-β* was no longer evident after 3.5 h of stimulation with *L. acidophilus* prior to addition of *H. pylori* 251. A similar but less-pronounced inhibitory effect was observed when the expression of *Il-12* was measured, since significant suppression was evident only until the *L. acidophilus* preincubation time of 2 h prior to addition of *H. pylori* 251 (*, $P <$ 0.01). Thus, our results suggest that *H. pylori*-mediated inhibition of *Ifn-β* and *Il-12* responses takes place after endocytosis and that cellular uptake and processing of *L. acidophilus* may be required for an induction of IFN-β and IL-12.

We then aimed to confirm this inhibitory effect on other cytokines and chemokines using RT-PCR. Expression levels of the T cell- and macrophage-recruiting chemokine gene *Cxcl10*, as well as those of the proinflammatory cytokine genes *Tnf-α* and *IL-1b*, were analyzed after 4 h (see Fig. S2 in the supplemental material). Our results show that the addition of *H. pylori* 251 to BMDMs preincubated with *L. acidophilus* is able to significantly downregulate the expression of *Cxcl10* and *Tnf-α* (*, $P < 0.01$). However, the expression of *IL-1β* was significantly upregulated by the addition of *H. pylori* 251 to BMDMs preincubated with *L. acidophilus* compared to results with *L. acidophilus* alone (*, $P < 0.01$), which is in accordance with our microarray results. Thus, we hereby demonstrate that *H. pylori* is able to inhibit the activation of cytokines that modulate an adaptive immune response but has an additive effect on IL-1β when added to BMDMs prestimulated with *L. acidophilus*.

Inhibition of IFN-β by *H. pylori* is independent of viability and the virulence factor CagPAI but dependent on the toxin VacA. To establish the role of the virulence factor CagPAI in blocking the expression of IFN-β, we added isogenic *H. pylori* 251 CagPAI mutant bacteria to BMDMs preincubated with *L. acidophilus* and measured the gene expression of *Ifn-β* after 4 h (Fig. 3A). The *H. pylori* 251 CagPAI mutant inhibited *Ifn-β* expression in BMDMs preincubated with *L. acidophilus* to a level equivalent to that of the wild-type *H. pylori* 251 strain, indicating that the inhibitory effect is independent of CagPAI. To determine whether bacterial viability is essential, we added heat-killed (HK) *H. pylori* 251 and HK 251 CagPAI mutant to BMDMs prestimulated with *L. acidophilus* and measured the level of IFN-β in the supernatants (Fig. 3B). Both HK *H. pylori* strains were capable of significantly blocking *L. acidophilus*-induced IFN-β (*, $P < 0.01$). Hence, our findings suggest that the inhibitory effect of *H. pylori* on IFN-β expression in *L. acidophilus*-stimulated BMDMs is dependent neither on bacterial viability nor on the presence of CagPAI.

Our microarray analysis revealed that key regulators of the Rho, Rac, and Cdc42 GTPases are strongly suppressed upon stimulation of BMDMs with *L. acidophilus* and *H. pylori* in combination. Entry of the *H. pylori* VacA toxin into epithelial cells is dependent on pinocytosis and controlled by the Cdc42 GTPase (31). Furthermore, in epithelial cells, VacA utilizes the Rac1 GTPase to induce cell vacuolation in host cells (32). We therefore speculated that the *H. pylori* VacA toxin may also play a role in macrophages and may be implicated in the inhibition of *Ifn-β* (Fig. 3C). Our hypothesis was confirmed, since an isogenic *H. pylori* 251 *vacA* mutant, in contrast to the wild-type strain, was unable to block *Ifn-β* when added to BMDMs prestimulated with *L. acidophilus* ($P < 0.01$). To recapitulate that VacA is essential for the immunosuppression of *L. acidophilus*-induced IFN-β, we added bacterial-free supernatants (SN) from wild-type *H. pylori* 251 in various dilutions to BMDMs prestimulated with *L. acidophilus* (see Fig. S3A in the supplemental material). Our finding was confirmed, since the VacA-containing supernatant from wild-type *H. pylori* 251 strongly inhibited the IFN-β response induced by *L. acidophilus*. In contrast, bacterial-free supernatant from the *H. pylori* 251 *vacA* mutant was not capable of suppressing IFN-β produced by *L. acidophilus*-stimulated macrophages (see Fig. S3B).

In epithelial cells, VacA is known to disrupt vesicular trafficking due to formation of anion-selective channels in the membranes of late endocytic compartments (33). We therefore sought to determine whether a similar mechanism is employed in mac-

rophages and whether phagosomal processing of *L. acidophilus* is necessary for the induction of IFN-β. BMDMs were preincubated for 30 min with the endosomal acidification blockers chloroquine and bafilomycin A1 prior to stimulation with *L. acidophilus* (Fig. 3D). Viability of the cells was verified via trypan blue staining (data not shown). Both inhibitors completely blocked the release of IFN-β, indicating that phagosomal maturation and processing of *L. acidophilus* are required for the production of IFN-β (*, $P < 0.01$).

***H. pylori* redirects the endocytic pathway of *L. acidophilus*.** Since our previous experiment revealed that phagosomal processing of *L. acidophilus* is required to trigger the release of IFN-β in macrophages, we hypothesized that *H. pylori* redirects the intracellular processing and degradation of *L. acidophilus*. To determine the intracellular location of *H. pylori* and *L. acidophilus*, we applied deconvolution microscopy and analyzed the uptake of the bacteria with the early endosome marker EEA1 (34) and the late endosome and lysosome marker LAMP-1 (35). Since we have shown that *H. pylori* is able to block the induction of *Ifn-*β at a very early stage (Fig. 2B), we added *L. acidophilus* NCFM to BMDMs and analyzed the localization of the bacterium after 2 h. As can be observed in Fig. 4A, a large proportion of intracellular *L. acidophilus* bacteria did not colocalize with either EEA1 or LAMP-1 compartments. Indeed, we determined that after 2 h of incubation, approximately only 7% and 30% of intracellular *L. acidophilus* NCFM were contained within endosomal and lysosomal compartments, respectively (Fig. 4C). The remainder of bacteria were not associated with either marker (*, $P < 0.05$; **, $P < 0.01$). In contrast, we detected the formation of megasomes and a significant increase in *L. acidophilus* colocalized in the endosomal and lysosomal compartments (19% and 50%, respectively, after 2 h incubation) when *H. pylori* was added, compared to results for BMDMs that were stimulated with *L. acidophilus* alone (Fig. 4B and C). Figure 4D presents a magnified image of *L. acidophilus* localized in lysosomes (white arrow) in either the presence or absence of *H. pylori*. Our results indicate a greater release of *L. acidophilus* from the phagocytic compartments in the absence of *H. pylori*. We suggest that the addition of *H. pylori* leads to an accumulation of *L. acidophilus* in the endosomal and lysosomal compartments, thereby preventing the induction of IFN-β by *L. acidophilus*.

DISCUSSION

In the present work, we aimed to investigate whether *L. acidophilus* is able to modulate the inflammatory response induced by *H. pylori* in bone marrow-derived macrophages (BMDMs) by examining the cellular and molecular mechanisms involved in uptake and processing of these organisms. Whole-genome microarray analysis revealed that while *L. acidophilus* induces T-cell recruiting chemokines (Cxcl10 and Cxcl11), proinflammatory (IL-1β and IL-6), and Th1-skewing cytokines (IFN-β and IL-12), *H. pylori* predominantly induced cytokines indicative of innate immune responses (IL-1β and IL-1α) and neutrophil-recruiting chemokines (Cxcl1, Cxcl2, and Cxcl3). Most importantly, microarray analysis revealed that when *H. pylori* is added to BMDMs prestimulated with *L. acidophilus*, Th1-skewing cytokines induced by *L. acidophilus* are strongly inhibited. Real-time PCR confirmed that *H. pylori* blocks the gene expression of IFN-β, IL-12, Cxcl10, and TNF-α, while it has an additive effect on the cytokine IL-1β. Thus, our results show that *H. pylori* prevents the production of factors induced by *L. acidophilus* in macrophages that contribute to initiating the adaptive immune response.

Interestingly, our microarray analysis also revealed that addition of *H. pylori* to macrophages prestimulated with *L. acidophilus* had a major impact on regulators of cytoskeletal arrangements and actin filament depolarization, since the genes encoding Pdrx2 and Pdxp were strongly induced upon incubation with both bacteria. In contrast, the genes *Rgs1/2*, *Fgd2*, and *Dock8*, regulators of the Rho, Rac, and Cdc42 GTPases, which are molecular switches controlling the organization and dynamics of the actin cytoskeleton, were strongly suppressed. It is well established that many bacterial pathogens regulate host trafficking pathways by the selective inclusion or retention of GTPases in host cells during infection and use these important cellular regulators as part of their overall virulence strategy (36, 37). Thus, our data suggest that *H. pylori* modifies GTPases in macrophages to promote bacterial persistence in the host.

Expression of constitutively active Rac1, a member of the Rho family of small GTPases, potentiates the activity of the *H. pylori* VacA toxin in two different epithelial cell lines (SCC12F keratinocytes and MDCK cells) (32). Furthermore, pinocytosis of VacA is regulated by the Cdc42 GTPase in HeLa and gastric AGS cells (31). Hence, we postulated that the same mechanism may be utilized in macrophages and that the *H. pylori* VacA toxin is involved in the immune-inhibitory effect observed. Indeed, the addition of *H. pylori vacA* mutant and VacA-containing supernatant from wild-type *H. pylori* 251 to *L. acidophilus*-stimulated BMDMs showed that VacA is essential for the inhibition of IFN-β responses.

Upon internalization in macrophages, *H. pylori* induces the fusion of the individual phagosomes, resembling giant multivesicular bodies (13), an effect that we observed in our microscopy (Fig. 4B). Since VacA leads to cell vacuolation in the cell membranes of the late endosomal compartments in epithelial cells (33), we used the endosomal acidification blockers chloroquine and bafilomycin A1 to determine whether phagosomal maturation and processing of *L. acidophilus* is required. Both inhibitors completely abrogated the expression of IFN-β in BMDMs stimulated with *L. acidophilus*, indicating that both phagocytic uptake and phagolysosomal maturation is a prerequisite for IFN-β signaling in response to this bacterium. Our microscopy data showed that *H. pylori* changes the endosomal transport of *L. acidophilus* from the endosomal and lysosomal compartments in BMDMs, suggesting that a specific endocytotic pathway is a key step for the induction of IFN-β.

The immunosuppressive ability of *H. pylori* is further corroborated by our microarray data. We identified that Socs3, a negative regulator of cytokine signaling, was among the most strongly expressed genes in macrophages stimulated with *H. pylori* (see Table S2 in the supplemental material). Socs3 is upregulated by several viruses, e.g., herpes simplex virus 1 (HSV-1) (38), hepatitis virus C (HCV) (39), influenza A virus (40), human immunodeficiency virus type 1 (HIV-1) (41), severe acute respiratory syndrome (SARS) virus (42), and respiratory syncytial virus (RSV) (43, 44), resulting in the inhibition of the type I interferon signaling pathway. Hence, the expression of *Socs3* in macrophages stimulated with *H. pylori* might be an additional mechanism utilized by this pathogen to suppress cytokine signaling and thereby the activation of adaptive immunity.

We therefore suggest that the *H. pylori* VacA toxin causes a redirection of the endocytic pathway in macrophages that pre-

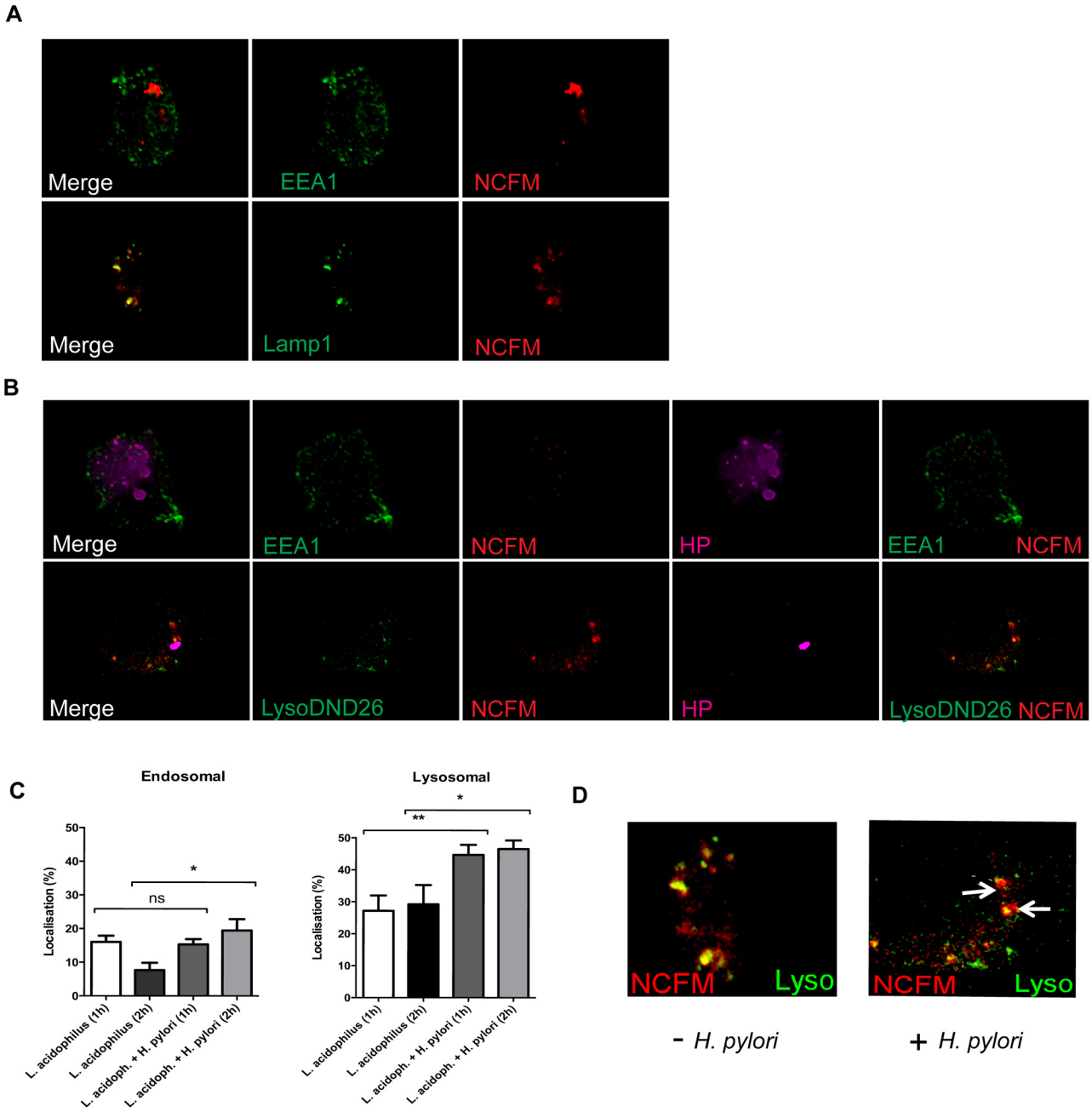

FIG 4 *H. pylori* redirects the endocytotic pathway of *L. acidophilus*. Phagocytic uptake of bacteria into early endosomes and lysosomes is shown. (A) BMDMs were exposed to red-labeled *L. acidophilus* NCFM for 2 h. Cells were either labeled with an EEA1 antibody for early endosomes or transfected with Lamp1-YFP for lysosomes. (B) As per panel A, with the addition of labeled or unlabeled *H. pylori* 251 (magenta). Cells were prelabeled with Lysotracker DND-26 to label lysosomes, incubated for 2 h, fixed, permeabilized, and stained with the EEA-1 antibody or anti-*H. pylori* serum for unlabeled *H. pylori* (HP). (C) Percentage of the total *L. acidophilus* NCFM located in the endosomal compartment or lysosomal compartment after 1 h and 2 h of incubation (with or without *H. pylori* 251). SEM are shown (*, $P < 0.05$; **, $P < 0.01$). (D) Magnified image of *L. acidophilus* NCFM (red) localized in lysosomes labeled with Lyso-DND26 (green) (2-h time point). Yellow indicates colocalization (white arrow) of signal or *L. acidophilus* NCFM located within the lysosomes. Data are representative of three independent experiments.

vents the activation of adaptive immunity, exemplified by the Th1-skewing commensal *L. acidophilus* in our present study. Collectively, this suppression will limit the effectiveness of *L. acidophilus* NCFM as a probiotic during treatment of *H. pylori*-induced inflammation, symptoms, or eradication from the host. Although additional investigations are required to determine how *L. acidophilus* may encounter macrophages *in vivo*, it is plausible that this occurs during inflammatory states. Identification of the molecular mechanisms underlying VacA action will lead to a better understanding of the pathogenesis of *H. pylori*-associated diseases, as they are possible targets for the treatment of *H. pylori* infection and for vaccination.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and preparation. The Gram-positive lactic acid bacterium *Lactobacillus acidophilus* NCFM (Danisco, Copenhagen, Denmark) was grown anaerobically overnight at 37°C in de Man Rogosa Sharp (MRS) broth (Merck, Darmstadt, Germany). The Gram-negative bacterium *Helicobacter pylori* was grown on solid agar and liquid broth as previously described (45). The following strains and mutants were used: the clinical isolate *Helicobacter pylori* 251 (46) and the *Helicobacter pylori* 251 CagPAI deletion mutant (45). The *Helicobacter pylori* 251 VacA (HP0887) mutant was constructed in this study by natural transformation using the plasmid pCTB8::Km (47), a kind gift from Tim Cover (Vanderbilt University, Nashville, TN). Confirmation of allelic exchange of VacA with *vacA*::Km in the 251 *vacA* mutant was performed by PCR. Heat killing of *H. pylori* was performed by incubating *H. pylori* at 60°C for 20 min and confirming the bacteria were no longer viable by testing their growth on solid horse blood agar as previously described (45).

Generation of murine macrophages from bone marrow progenitor cells. C57BL/6 mice were bred and housed under specific-pathogen-free (SPF) conditions within the animal facility of Monash Medical Centre. Mice were free of all *Helicobacter* species. All animal experiments were approved by and performed in accordance with the Monash Medical Centre Animal Ethics Committee. Female mice were used in these studies because they have been reported to be more susceptible to *Helicobacter*-induced pathology than male mice (48).

For the generation of murine bone marrow-derived macrophages (BMDMs), bone marrow from female 6- to 10-week-old C57BL/6 mice was flushed out from the femur and tibia and washed in phosphate-buffered saline (PBS). The cells were resuspended in RPMI 1640 medium (Gibco, Life Technologies, Singapore) containing 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Thermo Electron, VIC, Australia) supplemented with 1% penicillin-streptomycin (Gibco), 1% GlutaMAX (Gibco), and 20% of growth supernatant of L929 cells. The cells were seeded at a concentration of 7×10^6 cells in 10-cm^2 nontreated cell culture plates (Nunc, Roskilde, Denmark) in 10 ml of conditioned medium and incubated at 37°C in 5% CO_2. On day 4, 10 ml of complete medium containing 20% L929 cell-conditioned medium was added. On day 6, cells were harvested in conditioned medium, and 1×10^6 cells were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark). The next day, the medium was removed, and 400 μl of fresh RPMI 1640 medium (without antibiotics and FCS) was added.

Stimulation of murine bone marrow-derived macrophages with bacteria or bacterial-free supernatants. Overnight cultures of *L. acidophilus* and *H. pylori* were harvested by centrifugation at 1,250 $\times$ *g* for 10 min at 4°C and washed twice in sterile PBS. Bacteria were resuspended in RPMI 1640 medium (without FCS and antibiotics) and added (100 μl/well) to the BMDMs at an MOI of 1:1 (or medium, for a final volume of 500 μl/well). Bacterial-free supernatants (SN) from *H. pylori* 251 and the *H. pylori* 251 *vacA* mutant were prepared as previously reported (49). In brief, *H. pylori* grown for 24 h on agar plates was collected in 1 ml of sterile distilled water, vortexed for 30 s, and incubated at room temperature for 30 min. Bacteria were removed by centrifugation, and the supernatant was filter sterilized using a 0.2$-\mu$m filter. The VacA toxin contained within the water extracts was activated using hydrochloric acid, and the pH of the supernatant was subsequently neutralized prior to addition to BMDMs as previously described (50). Supernatants were added to BMDMs at various dilutions to a final volume of 500 μl/well. The cell cultures were incubated at 37°C in 5% CO_2. In the inhibitor experiments, BMDMs were preincubated for 30 min with the endosomal acidification blockers chloroquine (final concentration, 20 μM) and bafilomycin A1 (final concentration, 25 μM) prior to stimulation with *L. acidophilus*. Both inhibitors were purchased from InvivoGen (San Diego, CA).

Microarray analysis. Immature murine BMDMs from three female 6-week-old C57BL/6 mice were incubated with *L. acidophilus* NCFM, *H. pylori* 251, or *H. pylori* 251 added to BMDMs prestimulated with *L. acidophilus* NCFM for 1 h. The BMDMs were harvested 5 h after addition of *H. pylori*, and RNA was extracted using a Qiagen RNeasy minicolumn with on-column DNase digestion. For each sample, 200 ng of RNA was Cy3 labeled with a one-color low-RNA-input quick amplification kit (Agilent). The resulting cRNA was fragmented and hybridized to Agilent whole-mouse-genome expression microarrays (G4122F), and the slides were washed according to the manufacturer's instructions. Slides were scanned immediately after washing using an Agilent DNA microarray scanner (G2505B). Feature extraction was performed with Agilent Feature Extraction 9.5 software using default parameters excluding nonuniform flagged outliers. Percentile normalization and differential gene expression analysis were performed using the program GeneSpring GX 11 (Agilent Technologies).

RNA extraction and quantitative real-time PCR analysis. Murine BMDMs were harvested and homogenized by using a QIAshredder instrument (Qiagen, Australia), and RNA was extracted using the RNeasy Plus minikit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined with a NanoDrop instrument (Thermo, Wilmington), and 500 ng of total RNA was reverse transcribed with the TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA) using random hexamer primers according to the manufacturer's instructions. The obtained cDNA was stored in aliquots at -80°C. The following 6-carboxyfluorescein (FAM)-labeled TaqMan gene expression assays were purchased (Applied Biosystems): Cxcl10 (assay identifier [ID] Mm99999072_m1), IFN-β (assay ID Mm00439546_s1), IL-12b (p40) (assay ID Mm00434174_m1), TNF-α (assay ID Mm00443258_m1), IL-1β (assay, ID Mm00434228_m1), and 18S (assay, ID 4319413E). The amplifications were prepared in a total volume of 10 μl containing 1$\times$ TaqMan universal PCR master mix (Applied Biosystems) and diluted cDNA. All PCR reactions were carried out in triplicate in MicroAmp optical 384-well reaction plates (Applied Biosystems). The cycling parameters were initiated by 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60°C for 1 min using the 7900HT fast real-time PCR system (Applied Biosystems). The amplifications were normalized to the expression of 18S. Relative transcript levels were calculated, applying the $2^{-\Delta\Delta CT}$ method.

Cytokine quantification by ELISA. Protein production of IFN-β was quantified by enzyme-linked immunosorbent assay (ELISA) in the cell culture supernatants using rat anti-mouse IFN-β antibody (7F-D3; Abcam, USA) followed by rabbit anti-mouse IFN-β antibody (PBL Biomedicals, USA) for detection. Recombinant IFN-β was used as a standard (made in-house). IL-12p40 (OptEIA kit; BD, USA) was analyzed in the cell culture supernatants as per the manufacturer's instructions.

Microscopy. Immature murine BMDMs were seeded at a density 2×10^5 cells on 12-mm coverslips (Menzelglass, Germany) in 24-well tissue culture plates (Nunc) in a total volume of 500 μl. LAMP-1 yellow fluorescent protein (YFP) (concentration, 100 ng/1 $\times$ 10^5 cells; Rohan Teasdale, Institute for Molecular Bioscience, University of Queensland) was transfected into half of the wells containing BMDMs using the jetPEI macrophage *in vitro* DNA transfection kit (Polyplus Transfection SA, France), according to the manufacturer's instructions. The next day, medium was removed, and 200 μl of fresh RPMI 1640 medium (without antibiotics and FCS) was added. *L. acidophilus* NCFM was labeled red with the dye Dil (Vybrant cell-labeling solutions; Molecular Probes, Life Technologies, USA), and *H. pylori* 251 was labeled green with the dye DiO (Vybrant cell-labeling solutions) according to the manufacturer's protocol. The bacteria (or medium) were added (100 μl/well) to the BMDMs at an MOI of 1:1 (total volume, 400 μl). To determine the uptake of the bacteria in the early endosomes, Dil-labeled *L. acidophilus* NCFM and DiO-labeled *H. pylori* 251 were added to the BMDMs. The medium was removed, the cells were rinsed with PBS, fixed in 20% Formalin for 20 min, and stored at 4°C in PBS. For antibody labeling, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked in 5% bovine serum albumin (BSA)-PBS for 30 min, and labeled with the early endosome marker EEA1 antibody (Abcam, United States). For lysosomal

studies with coinfection, Dil-labeled *L. acidophilus* NCFM and unlabeled *H. pylori* 251 were added to the BMDMs prelabeled with LysoTracker green DND-26 (Molecular Probes, Life Technologies, USA) according to the manufacturer's instructions. Antibody labeling with purified anti-*H. pylori* serum (in-house) was performed as described previously (45). Cells were visualized by both confocal laser microscopy (Leica SP5) and wide-field deconvolution microscopy (Deltavision core system; Applied Precision, USA). Images are presented as a two-dimensional (2D) maximum-intensity projection or as single-plane images.

Statistical analysis. The GraphPad Prism software program, version 4.03 (GraphPad Software, San Diego, CA), was used, and one-way analysis of variance (ANOVA) and Bonferroni tests were applied ($P < 0.01$). The microarray data were analyzed applying one-way ANOVA with Benjamini-Hochberg multiple testing correction ($P < 0.01$). Colocalization studies were performed using the built-in feature in the Bitplane Imaris 7.5 software program with a threshold of background plus 2 standard deviations (SDs). Colocalization data were accumulated using the GraphPad Prism program as mentioned previously, and an unpaired *t* test was used to determine significance (*, $P < 0.05$; **, $P < 0.01$).

Microarray data accession number. The data generated in this work have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE42622.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00609-12/-/DCSupplemental.

Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Figure S1, TIF file, 1 MB.
Figure S2, TIFF file, 0.5 MB.
Figure S3, TIF file, 1.9 MB.

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